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Molecular Cloning, Bioinformatics Analysis and Transcriptional Expression of Virulence-related Gene (*exsA*) of *Vibrio alginolyticus*

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Abstract [Objectives] The purpose was to investigate the function and mechanism of *exsA* gene in type III secretion system of *Vibrio alginolyticus*. [Methods] The full length of *exsA* was cloned using molecular biology techniques to analyze its biological information. Fluorescence quantitative PCR technology was used to analyze the expression of *exsA* after different media stress. [Results] The *exsA* gene contains an open reading frame (ORF) of 861 bp, encoding 286 amino acids. The physico-chemical analysis shows that the molecular structural formula is $C_{1442}H_{2267}N_{393}O_{441}S_{12}$, the theoretical molecular weight is 32.549 kD, the theoretical *pI* value is 6.0, and the protein is non-hydrophilic and unstable. The gene does not contain a transmembrane region, and there is no obvious signal peptide. The prediction result of protein subcellular localization shows that the protein is inside the cell. The deduced amino acid sequence and constructed phylogenetic tree show that *V. alginolyticus* has a close relationship with *Vibrio antiquarius*. The qPCR results show that the expression level of *exsA* in different media is different, highest in TSB medium containing bile salts, followed by DMEM medium, and lowest in ordinary TSB medium. [Conclusions] The gene sequence, molecular structure and isoelectric point of *exsA*, as well as its expression in three different media were obtained.

Key words *Vibrio alginolyticus*, *exsA*, Type III secretion system, Bioinformatics analysis

1 Introduction

Vibrio alginolyticus (Vibrionaceae; *Vibrio*) is a halophilic and facultative anaerobic gram-negative bacterium. It is a common marine pathogen. The reproduction and growth of the bacterium has certain requirements for pH (6–9) and temperature (17–35 °C). In 1973, it became clear that *V. alginolyticus* could be pathogenic to humans, and by contaminating food, it causes people's diarrhea and wound infection. *V. alginolyticus* is a conditional pathogen, and is very harmful to China's aquaculture. When the aquaculture environment is bad and the immune function of aquatic animals is low, *V. alginolyticus* is prone to outbreak. In particular, it can make turbot, large yellow croaker, grouper, etc. get ill^[1], and can also infect shrimp^[2–4] and shellfish. Therefore, in recent years, many researchers have begun to study the molecular biology of *V. alginolyticus* to find out the prevention and control methods to solve the harm of *V. alginolyticus* to aquaculture.

V. alginolyticus has no spore or capsule, but planktonic *V. alginolyticus* can adhere to the surface of media and produce a large number of extracellular products to package and aggregate individual colonies into micro colonies. With the continuous in-

crease of extracellular products, individual microcolonies gradually connect to form a stable biofilm, so *V. alginolyticus* can resist bad environments and multiply^[5–7]. The pathogenic factors of *V. alginolyticus* are mainly divided into four categories: adhesin, outer membrane proteins (OMPs), extracellular products (ECP) and siderophores.

V. alginolyticus contains eight different protein secretion systems, named T1SS-T8SS^[8]. T3SS is a non-signal peptide-dependent secretion pathway, which is in the shape of a "needle". It is mainly composed of apparatus proteins, translocon proteins, effector proteins, regulation proteins and chaperones. The infection process of T3SS virulence system is very complicated. The whole process is completed through interaction and tight regulation of about 40 proteins and a secretion channel with a diameter of about 2 nm^[9]. The principle is to deliver the effector protein to the host cell to induce pathological changes such as rapid apoptosis or self-phagocytosis, rounding and osmotic swelling of the host cell^[10]. The study results of Zhou Xiaohui^[11–12] show that the regulation of T3SS1 gene expression in *Vibrio parahaemolyticus* is controlled by the interaction between ExsA, ExsD and ExsE; and ExsA is necessary to induce T3SS1 gene transcription, and it directly interacts with the promoter sequence of the T3SS1 gene and participate in the positive regulation of Vp1656 expression and secretion. However, Gao Ruxia^[13] found that the ExsA of *Pseudomonas aeruginosa* can affect its activity, which in turn affects the toxicity of the bacterium. The research on the T3SS protein secretion system is constantly progressing, and studying the regulation mechanism of *V. alginolyticus* ExsA will inevitably provide a data basis for the study of the T3SS system.

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2 Materials and methods

2.1 Materials

2.1.1 Vector and strain. *V. alginolyticus* virulent strain HY9901 was isolated from diseased red snapper fish in Zhanjiang waters of Guangdong Province^[14]. The cloning vector pMD18-T was purchased from Takara Bio Inc.

2.1.2 Main reagents. ExTaq DNA polymerase was purchased from Takara Bio Inc. Bacterial genomic DNA extraction kit and DNA gel recovery kit were purchased from Tiangen Biotech (Beijing) Co., Ltd. The remaining reagents (analytically pure) were imported or domestically produced. The PCR primer synthesis and sequencing were completed by Sangon Biotech (Shanghai) Co., Ltd. The concentration of the antibiotic used (ampicillin, Ap) was 100 µg/mL.

2.2 Methods

2.2.1 Extraction of total DNA of *V. alginolyticus* strain HY9901. *V. alginolyticus* strain HY9901 was inoculated in TSB medium (2% NaCl) and shaking-cultured at 28 °C for 18 h. An appropriate amount of the bacterial liquid was transferred into a centrifuge tube and centrifuged at 10 000 rpm/min for 1 min, and the bacterial cells were collected. According to the kit instruction, genomic DNA was extracted and store at -20 °C for later use.

2.2.2 Cloning of *exsA* gene. A pair of primers was designed according to the full genome sequence of *V. alginolyticus* registered on Genbank (ACCESSION: GU074526) (upstream primer S: ATGGATGTGTCAGGCCAACTA; downstream primer A: TCATTTCGCGATAGCCACTTG). The extracted total DNA of *V. alginolyticus* strain HY9901 was used as a template. The PCR reaction conditions were as follows: pre-denaturation at 95 °C for 5 min; denaturation at 95 °C for 30 s, annealing at 65 °C for 30 s, 72 °C for 60 s, 32 cycles; extending at 72 °C for 10 min. After examined by 1.0% agarose gel electrophoresis, the PCR product was recovered with gel DNA recovery kit.

2.2.3 Sequencing of PCR products. In line with relevant instructions, the PCR product was aligned to the pET32a(+) vector, transformed into *Escherichia coli* DH5α competent cells, and screened on Ap+/LB plates containing IPTG/X-Gal, and positive clones were selected and sent to Sangon Biotech (Shanghai) Co., Ltd. for sequencing.

2.2.4 Bioinformatics analysis of *exsA* of *V. alginolyticus* strain HY9901^[15-16]. Sequence homology comparison and similarity analysis were performed using NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). DNAMAN was used for amino acid homology analysis. ExPASy Proteomics Server (<http://web.expasy.org/protparam/>) was used to deduce amino acid sequence, determine open reading frame (ORF), predict calculated molecular weight (*M_w*) and theoretical isoelectric point (*pI*), *etc.* Through SignalP 4.0 Server (<http://www.cbs.dtu.dk/services/SignalP>), signal peptide sequence was predicted. TMHMM Server 2.0 (<http://www.cbs.dtu.dk/services/TMHMM>) was applied to predict the transmembrane domain. SoftBerry-Psite (

proloc) was used to predict the distribution of functional sites in the amino acid sequence. The promoter was analyzed with Promoter 2.0. PSORT II Prediction (<http://psort.hgc.jp/form2.html>) was adopted to predict subcellular location. Using Clustal 2.0 and MEGA 5.0, phylogenetic tree was constructed with neighbor-joining method. SWISS-MODEL (<http://www.swissmodel.expasy.org/>) was used to construct three-dimensional structure. Kegg (<http://www.genome.jp/kegg/>) was used to analyze the pathways involved. In the STRING database (<http://string.embl.de/>), protein network interactions were searched.

2.2.5 B cell epitope prediction of ExsA of *V. alginolyticus* strain HY9901. The Chou-Fasman method was used to predict the secondary structure of the protein. Parameters were predicted separately with Kyte-Doolittle (hydrophilicity), Karplus-Schulz (flexibility), Emini (surface accessibility) and Jameson-Wolf (antigenicity). The prediction result of each parameter was analyzed. Finally, combined with DNASTAR software, the B cell epitope of ExsA protein of *V. alginolyticus* strain HY9901 was determined.

2.2.6 Expression differences of *exsA* gene in different media. The expression of *exsA* gene in different media was determined using quantitative real-time reverse transcription PCR (qRT-PCR).

V. alginolyticus strain HY9901 with *OD*₆₀₀ of 0.5 was inoculated in DMEM (high sugar medium), 0.05% bile salt TSB and ordinary TSB at a ratio of 1:100, respectively. After culturing at 28 °C for 12 h, bacterial liquids were collected. RNA was extracted to synthesize cDNA for qRT-PCR reaction^[17]. Based on the conservative sequence of *exsA* gene, specific primers were designed for qRT-PCR analysis. The 16S rRNA gene was selected as the control gene. SYBR Green I was used as the marker. The primers used in the PCR reaction are shown in Table 1.

Table 1 Primers for quantitative real-time PCR

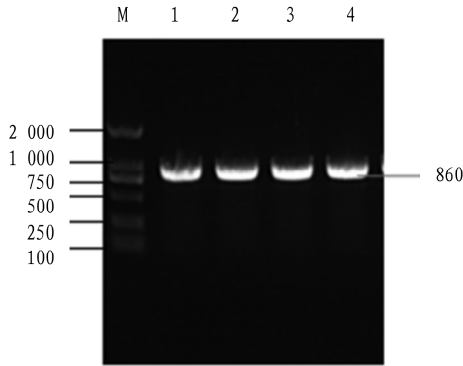
Primer	Sequence (5'-3')
RT- <i>exsA</i> -F	GATGGGCATACAAAGCCG
RT- <i>exsA</i> -R	CACATAATGGCATCAGGCTA
RT-16Sr RNA-F	TTGCGAGAGTGAGCGAATCC
RT-16SrRNA-R	ATGGTGTGACGGCGGTGTG

3 Results and analysis

3.1 Cloning of *exsA* gene A specific band of approximately 861 bp was obtained by PCR amplification (Fig. 1). The sequencing results show that the *exsA* gene contains an open reading frame (ORF) of 861bp, encoding 286 amino acids (Fig. 2). The accession number in GenBank is MN385414.

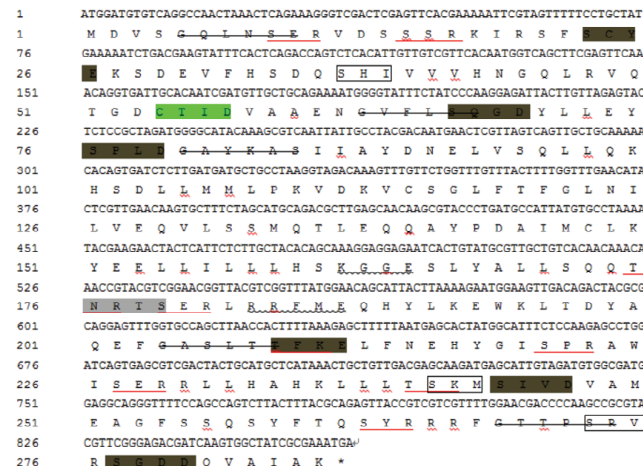
3.2 Analysis of physical and chemical properties of ExsA ExPASy software was used to analyze the ExsA protein of *V. alginolyticus* strain HY9901. The results show that the total number of atoms is 4 555, the molecular structure is C₁₄₄₂H₂₂₆₇N₃₉₃O₄₄₁S₁₂, the theoretical molecular weight is 32.549 kD, the theoretical *pI* value is 6.0, the instability coefficient is 53.96 (greater than the threshold value of 40, indicating that the properties are unstable, that is, the protein is unstable), the fat coefficient is 89.30, the

overall average coefficient of hydrophilicity is -0.278 , the total number of acidic amino acid residues (Asp + Glu) is 35, the total number of basic amino acids (Arg + Lys) is 30, and the N-terminal is methionine (Met). The half-life of expression in yeast and *E. coli* is greater than 20 and 10 h, respectively, and the half-life of expression in mammalian reticulocytes *in vitro* is 30 h.



Note: M, DL 2000 DNA marker; 1–4, PCR products.

Fig. 1 Cloning of *exsA* gene



Note: * represents terminator; the light gray shade is the N-glycosylation site; the red underlined part is the phosphorylation site of protein kinase C; the dark gray shade is the phosphorylation site of casein kinase II; the underlined wavy line represents the phosphorylation site of tyrosine kinase; the strikethrough represents the myristoylation site; the green shade is the prenyl binding site; and the box represents the C-terminal targeting signal site of microbody.

Fig. 2 Nucleotide sequence and encoded amino acid sequence of *exsA* gene

3.3 Sequence analysis of ExsA SignalP 4.1 Server was used to predict the N-terminal signal peptide structure of ExsA amino acid sequence, and no signal peptide was found between amino acids. The prediction results of TMHMM Server v. 2.0 show that no transmembrane domain was found. The prediction results of SoftBerry-Psite (predict protein) show that the amino acid sequence contains one N-glycosylation site, 10 protein kinase C phosphorylation sites, 6 casein kinase II phosphorylation sites, 3 tyrosine kinase phosphorylation sites, 5 N-myristoylation sites, one isopentenyl binding site, and 3 microbial C-terminal targeting signals. The prediction results of protein subcellular localization

show that the probabilities of ExsA being distributed in plasma membrane, cytoplasm, vacuole, mitochondrion, endoplasmic reticulum, vesicle and nucleus are 4.3%, 43.5%, 4.3%, 13.0%, 4.3%, 13.0% and 17.4%, respectively.

3.4 Homology and evolution analysis of *exsA* BLAST analysis found that *V. alginolyticus exsA* has high homology with *exsA* of other species, and the homology with *Vibrio antiquarius* is up to 97%. Similarity analysis shows that *exsA* in *Vibrio* is conserved (Fig. 3).

Using the neighbor-joining method of MEGA 5.0, a phylogenetic tree was constructed by combining the deduced ExsA amino acid sequence of *V. alginolyticus* with those of other microorganisms. The results show that the ExsA of *V. alginolyticus* strain HY9901 and *V. antiquarius* belong to the same cluster, indicating that they are closely related (Fig. 4), consistent with the traditional morphological and biochemical characteristics classification results.

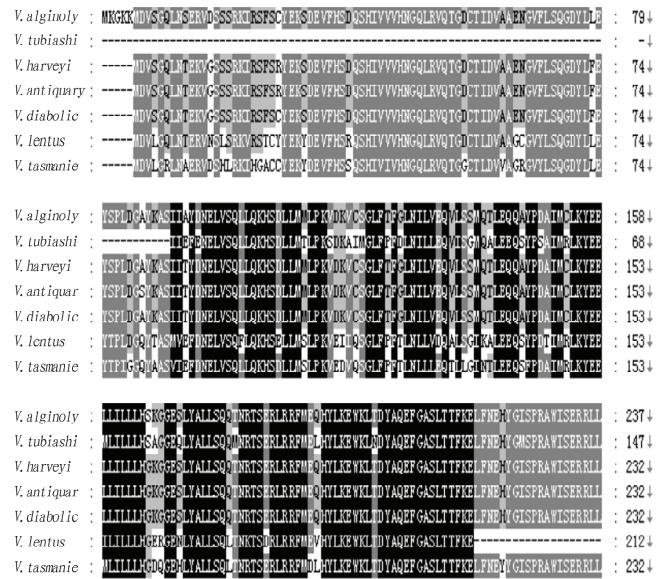


Fig. 3 Homology analysis of deduced amino acid sequences of *exsA* gene

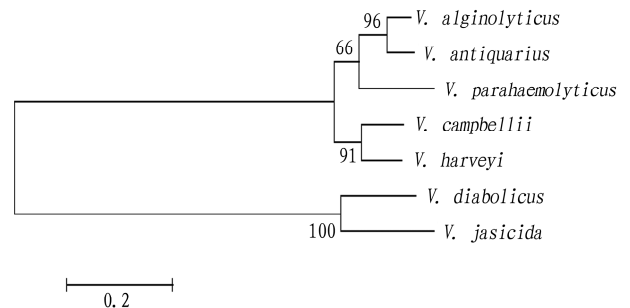


Fig. 4 Phylogenetic tree of ExsA amino acid sequences based on NJ method

3.5 Prediction of functional domain and secondary structure of ExsA SMART was used to predict the functional domain of ExsA. It is found that ExsA has a main functional domain HTH_GNTR (Fig. 5). The secondary structure of ExsA was predicted using SOPMA. The results show that in the secondary structure of

ExsA, α -helix, random coil, extended strand and β -fold account for 44.06%, 22.38%, 20.28% and 13.29%, respectively (Fig. 6).

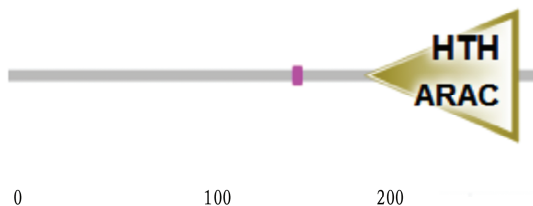
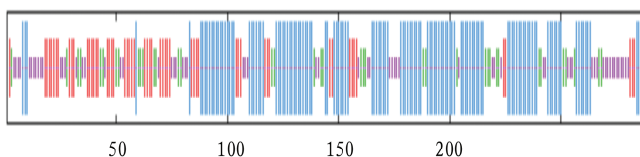


Fig. 5 Domain prediction of ExsA protein



Note: blue line, α -helix; green line, β -fold; red line, extended strand; purple line, random coil.

Fig. 6 Secondary structure prediction of ExsA protein

3.6 Subunit structure of ExsA The amino acid sequence of ExsA was submitted to the SWISS-MODEL program to automatically search for homologous proteins as templates, and the tertiary structure model of ExsA single subunit was obtained (Fig. 7).

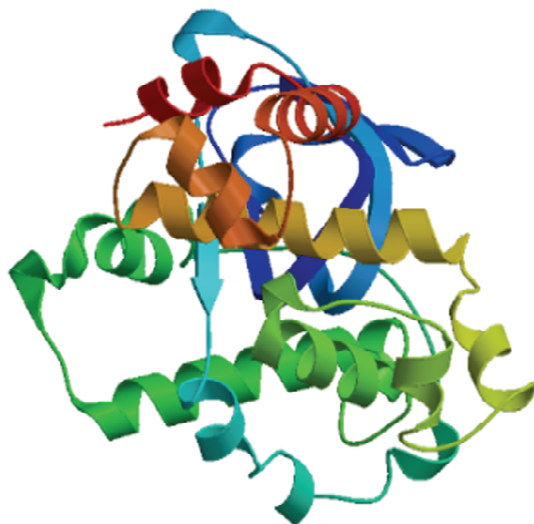


Fig. 7 Tertiary structure model of single subunit of ExsA

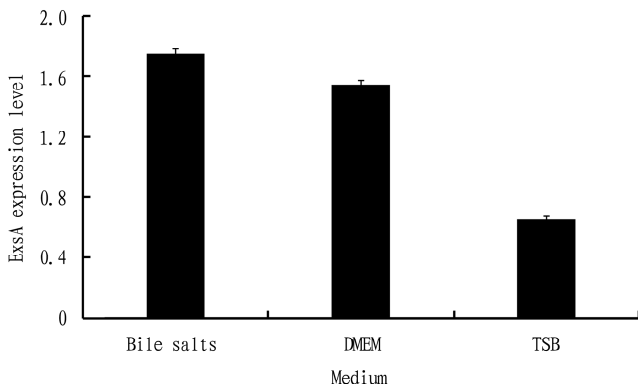


Fig. 8 Expression level of *exsA* gene in different medium

3.7 Expression analysis of *exsA* Using quantitative real-time PCR technology, the expression of *V. alginolyticus exsA* after different media stress was determined. The result is shown in Fig. 8. The *exsA* gene could be expressed in three different media, but the expression level was different, highest in TSB medium containing bile salts, followed by DMEM medium, and lowest in TSB medium.

4 Discussion

V. alginolyticus is an important pathogen that causes diseases in marine aquaculture economic animals, causing greater economic losses to the industry. Currently, the domestic prevention and treatment of vibriosis still mainly relies on chemical drugs and antibiotics. The resulting environmental pollution, bacterial resistance and drug residues are becoming more and more serious^[18]. Therefore, looking for the main protective antigens and cloning their genes to express subunit vaccines with stronger immunogenicity and lower side effects have gradually attracted the attention of scholars.

At present, the research on *V. alginolyticus* focuses on the isolation and identification of pathogens, pathological changes, *etc*^[19], but there are relatively few studies on its virulence-related genes. Research shows *V. alginolyticus* can produce adhesion factors, lipopolysaccharide, type III secretion system, outer membrane protein and other virulence factors^[20]. Studying the virulence-related genes of *V. alginolyticus* and finding an ideal immune protective antigen for the immune prevention and treatment of vibriosis is the key to solving the problem.

Bioinformatics analysis is an important method to analyze protein structure and function^[21]. It has been widely used in vaccine development^[22]. The prediction results of this study show that there is no signal peptide at the N-terminus of ExsA protein, so it can be inferred that ExsA protein is not a secreted protein. Protein phosphorylation can modify proteins^[23-24] and is the key to regulating the cell cycle. It is a form of molecular switch. Due to lack of endoplasmic reticulum and Golgi apparatus, ExsA protein in *V. alginolyticus* cannot be glycosylated and acylated. However, it has no effect on phosphorylation modification^[25].

With the development of bioinformatics, using biological software to predict and screen antigens has become a research hotspot^[26-27]. For a long time, to explore the common antigen of *Vibrio*, scholars' research on this bacteria is mostly related to infection-related enzymes^[28-29], attachment and colonization factors^[30-31], transporters^[32], *etc*. However, there are few reports on ExsA of *V. alginolyticus*. The amino acid sequence encoded by *exsA* gene is highly conserved, and its similarity with other *Vibrio* species is higher than 60%, which implies that the function of *exsA* gene is conserved. It is speculated that the ExsA protein of *V. alginolyticus* may be a common antigen of *Vibrio*, but it needs to be verified by prokaryotic expression, immunogenicity and immune protection, so as to find an effective common antigen for the prevention and treatment of vibriosis.

The phylogenetic tree constructed shows that *V. alginolyticus* strain HY9901 has a close relationship with *V. antiquarius*.

5 Conclusions

In this study, the *exsA* gene of *V. alginolyticus* was cloned.

The sequence analysis shows that *exsA* contains an open reading frame (ORF) of 861 bp, encoding a total of 286 amino acids. Its molecular structure is C₁₄₄₂H₂₂₆₇N₃₉₃O₄₄₁S₁₂, theoretical molecular weight is 32.549 kD, and theoretical *pI* value is 6.0. The protein does not contain a transmembrane region, is non-hydrophilic, and contains multiple sites. *V. alginolyticus* is found to be closely related to *V. antiquarius*. The ExsA protein of *V. alginolyticus* has a main functional domain HTH_GNTR. The results of structural prediction show that in secondary structure, α -helix, random coil, extended strand and β -fold account for 44.06%, 22.38%, 20.28% and 13.29%, respectively. In different media, the expression level of *exsA* is highest in TSB medium containing bile salts, followed by that in DMEM medium, and lowest in TSB medium.

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